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Pollutants as Developmental Toxicants in Aquatic Organisms

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Pollutants, by disrupting metabolic processes, can interfere with development, and, at critical periods of development, can act as teratogens. Such interference with normal development can be used as a bioassay. Some screening tests are based on this phenomenon.

As teratogens, pollutants are fairly nonspecific. Many different classes may elicit the same developmental responses. Mechanisms of teratogenicity include disruption of mitosis, interference with transcription and translation, metabolic disturbances in energy utilization, and nutritional deficits. These in turn interfere with cell interactions, migration, and growth.

In aquatic organisms, environmental conditions can be critical. Interactions of pollutant effects with salinity and with temperature have been reported. Interactions between toxicants have also been studied; both synergism and antagonism have been reported.

Most reports of teratogenesis have been qualitative. Quantitation has usually been in the form of percentages of embryos affected, but when severity of effect is indexed, more critical analysis is allowed. When effects of other developmental processes such as growth are analyzed, quantitation is readily achieved. Regeneration is an especially useful model of both differentiation and growth. These two components of regeneration can be separately analyzed. Dose-response relationships are readily apparent.

In comparison to mammalian embryos, the use of embryos of many aquatic species for testing toxicants has certain advantages, including lower cost and maintenance and shorter development times. They respond to many of the same teratogens. A special advantage is availability for continual examination during development so that abnormalities can be observed and recorded as they arise.

Introduction

Aquatic toxicologists have known for a long time that embryos and larvae are often the most sensitive stages in an animal's life cycle. For this reason, whole life cycle tests have often been replaced by early life stage tests (*1*). In these tests, organisms are exposed from the time of fertilization until some weeks after hatching. The data gathered are generally total hatching success and survival and growth of the larvae (*2*). These are parameters that are easy to screen in large-scale operations and do not require special experitse. These tests, while utilizing embryos, do not provide insight into specific teratogenic effects of the chemicals tested in either a qualitative or a quantitative way. On the other hand, from the point of view of a regulatory agency attempting to establish "safe levels" of a substance, the precise nature and degree of deformities produced is immaterial; what is important is the overall survival rate through the embryonic and early larval stages. Early life-stage tests were designed not as tests for developmental toxicants in particular, but as general toxicity screening methods.

There are, however, toxicants that exert their effects particularly on developing systems, and regulatory agencies are concerned about pollutants that might cause birth defects. The Environmental Protection Agency (EPA) has issued guidelines for risk assessment for suspected developmental toxicants (*3*). The term "developmental toxicity" is somewhat broader than "teratology," encompassing embryotoxicity, altered growth, and functional deficiency in the offspring (which may not be apparent until after birth), in addition to structural abnormalities. However, these EPA guidelines are concerned exclusively with mammalian developmental toxicity testing. In this paper, the term "teratology" will be used in the broad, rather than in the narrow sense. There have been, however, few studies on aquatic species which examined functional deficiency in the offspring.

There are bioassays being developed for environmental teratogens using nonmammalian forms. One such test is the FETAX system, using *Xenopus* embryos (*4–6*). A teratogenicity index comparing LC_{50} and EC_{50} (for malformations) can rate the relative teratogenic strength of substances. Compounds that are strong teratogens in this system also tend to be highly teratogenic in mammalian systems. Teratogenicity test systems being developed using aquatic organisms in-

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clude a planaria regeneration test, a Hydra reaggregation system (*7*), and a sea urchin embryo screening test (*8*).

In addition to the development of teratogen bioassays, there are many studies of reproductive and developmental effects of environmental pollutants on aquatic biota. In addition to studies of the nature and degree of abnormalities produced, there are attempts to analyze mechanisms of action of the teratogens. The effects studied include, of course, embryonic malformations, as well as effects on developmental processes that occur at later life stages, such as regeneration.

In the bioassays being developed, as well as in most of the experiments to be discussed in this paper (which will focus on fish), embryos are exposed to the toxicant after fertilization. However, embryos in the natural environment can be exposed to pollutants in two additional ways: via yolk which is synthesized during oogenesis by exposed females, and during the brief period between shedding of the gametes and elevation of the chorion. A few studies have shown that toxicants incorporated into the egg during oogenesis can produce malformations in the embryos that subsequently develop from those eggs (DDT, lathyrogenic agents, and zinc) (*9–11*). While in most cases the chorion can act as a barrier to partially protect the developing embryo from the toxic effects of the pollutant (*12,13*) there have been a few reports that have shown that dechorionated embryos were actually less susceptible to the toxic effects than embryos with an intact chorion (*14,15*). Recently, this has been explained for metal ions by Rombough (*16*) in relation to the Donnan equilibrium. Those cations that are electronegative (e.g., Zn^{2+}, Cd^{2+}, and Pb^{2+}) can readily penetrate the chorion (which acts as an ion exchanger) and are concentrated in the perivitelline fluid. Thus, embryos with a chorion are more susceptible to these ions than those without. The reverse is true of electropositive ions (e.g., Hg^{2+}, Cu^{2+}, Ag^{2+}); these bind to the chorion, allowing it to act as a barrier.

Teratogenic Effects

Teratogens tend to be fairly nonspecific in the nature of the defects that they cause (*17*). Although many different substances can produce the same kinds of deformities, the actual modes of action of the different chemicals may differ. General developmental mechanisms that can be disrupted and lead to abnormal development include: abnormal cell or tissue differentiation, excessive or inadequate cell death during development, inadequate cell migration, improper cellular communication, and disrupted metabolism (respiration, absorption, excretion, or secretion).

Fish embryos, in general, tend to become abnormal in certain ways. The most sensitive system appears to be the developing skeletal system, and flexures (scoliosis, lordosis) as well as stunting are seen in many species treated with a variety of teratogens. Another common set of abnormalities involves the developing circulatory system. Circulatory stasis, a failure of the heart tube to bend, and edema of the pericardial cavity are also commonly observed defects. The developing optical system is also very sensitive, and many investigators have observed optical malformations, such as microphthalmia and anophthalmia, as well as cyclopia and intermediate conditions of fusion of the two optic vesicles. Although not strictly a developmental anomaly, another phenomenon often observed in teratogen-exposed embryos is a general retardation of development. This decrease in developmental rate, sometimes seen as an arrest of development, may permit teratogens to act for a longer than normal time during sensitive ("critical") stages, and thus intensify the severity of the anomalies produced. Embryos of *Fundulus*, exposed continuously to the insecticides carbaryl or parathion, arrested their development at stage 22 or 24, depending on the time of initial exposure. If, however, they were transferred to clean water after 4 days, they could resume development, but most of them developed circulatory abnormalities (*18*). The period of arrest made them vulnerable to the production of the malformations. Developmental arrest in itself does not cause abnormalities upon recovery and the resumption of development. Laale and McCallion (*19*) found that homogenates of zebrafish embryos caused intact embryos to arrest their development at stage 17–18. These embryos showed no mitotic figures. Upon return to fresh water, the embryos resumed normal development. *Fundulus* embryos forced to develop anaerobically by cyanide or nitrogen arrested at high blastula. Upon release from the anaerobic conditions, normal development resumed (*20*). Therefore, it is not the developmental arrest itself but the continuous exposure to a teratogen at that critical time that causes the anomalies once development resumes. Conversely, environmental or inherent factors that enable embryos to develop more rapidly, spending less time at critical stages, can make them less susceptible to teratogenic influences (*21,22*).

Optic Malformations

Abnormalities in the development of the optic cups have been observed by many investigators. Dial (*23*) observed disorganized retinas, abnormal pigment distribution, and invasive blood sinuses in eyes of medakas treated with methylmercury. Wilson (*12*) found similar disturbances in embryos of herring, plaice, and sole treated with oil dispersants. Lonning (*24*) found reduced pigmentation and protruding lenses in oil-treated cod and flatfish embryos; these anomalies were produced when the embryos were treated for as little as 1 to 2 hr during cleavage stages. Similar optic cup abnormalities have been produced in *Xenopus* embryos by various fungicides (*25*). Microphthalmia and anophthalmia were observed in Atlantic silversides (*Menidia menidia*) embryos treated with insecticides (*26*) (Fig. 1) and in rainbow trout (*Salmo gairdneri*) embryos treated with benzo[a]pyrene (*27*). Reduced mitotic index and increased incidence of pyknotic cells in the optic cups were observed in the latter treated embryos. Thus, re-

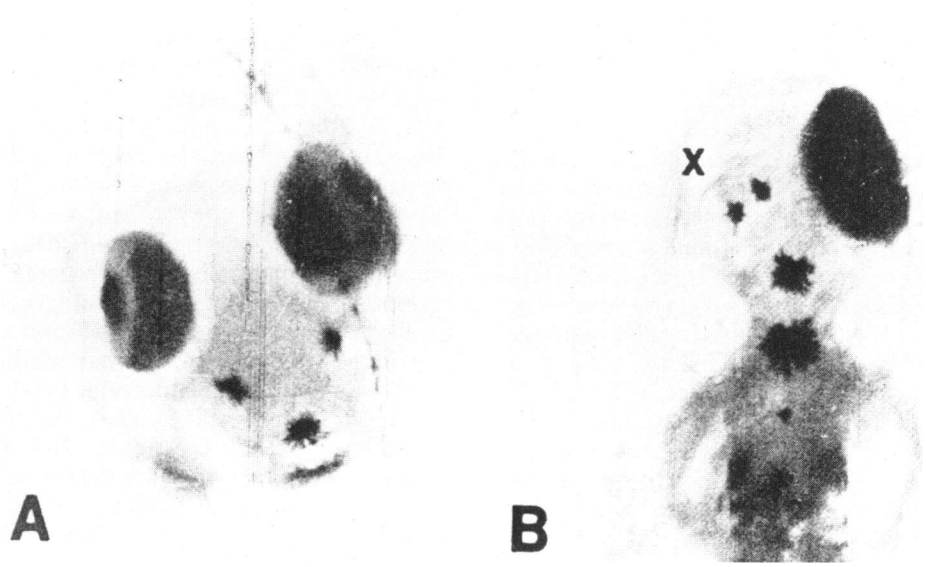

FIGURE 1. Photomicrograph of whole, fixed 2-week-old *Menidia menidia* embryos (approximately 20×): (*A*) control embryo; (*B*) embryo treated with the insecticide carbaryl (Sevin) at 10 μg/L; unilateral anophthalmia is demonstrated, with the site of the missing eye at X. Reproduced from Weis and Weis (*26*).

duced cell number in the eyes, due to the genotoxic action of the chemical, can account for the abnormally small eyes. Similarly, Perry et al. (*28*) have noted decrease in mitotic index and increased percentage of abnormal mitoses in methylmercury-treated embryos of *F. heteroclitus*. Embryos with more severe teratogenic responses also exhibited more severe mutagenic responses. Pelagic fish eggs can be used to monitor pollutant effects by studying the mitotic index and chromosome abnormalities in specimens collected from differentially polluted areas (*29*).

Many investigators, starting with Stockard, have noted a defect in forebrain development in which the eye rudiments converge, sometimes to the point of cyclopia. The cyclopic eye noted in $MgCl_2$- (*30*), $HgCl_2$- (*31*), and methylmercury-treated (*32*) Fundulus embryos, as well in ethanol-treated (*33*) *Brachydanio rerio* exhibits an unusually large optic cup and lens, since it is the result of the fusion of the two separate rudiments. The mechanism underlying the fusion of the optic vesicles is believed to be an inadequate induction of the forebrain, which then permits the two vesicles to approach one another in the anterior midline of the embryo. This anomaly, therefore, is not strictly an optic one, but its genesis involves a defect in craniofacial development.

Cardiac Malformations

Defects involving thin atrial and ventricular walls, failure of the heart to bend ("tube heart"), decreased circulation, and hemostasis, often accompanied by pericardial swelling and lack of blood pigment, have been observed often in fish embryos. Among the environmental pollutants producing these anomalies are alkaline pH in the Atlantic salmon, *Salmo salar* (*34*), cadmium in *S. gairdneri* (*35*), carbaryl in *Fundulus heteroclitus* (*18*), carbaryl, parathion and malathion in *Oryzias latipes* (*36*), mercury compounds in *Fundulus heteroclitus* (*31,32*), mercury in *Oryzias latipes* (*37*), lead in *Brachydanio rerio* (*13*), toluene in *O. latipes* (*38*), toluene in *Pimephales promelas* (*39*), 2,4,5-T in *O. latipes* (*40*), ethyl carbamate in *Brachydanio rerio* (*41*), and aflatoxin in *O. latipes* (*42*). It is believed that the pericardial edema is a result of a fluid imbalance caused by the retarded circulation (*17*). While the underlying mechanisms in the production of these cardiac abnormalities in fishes have not been ascertained, some work on bird embryos may reveal similar mechanisms. Proctor and Casida (*43*) showed that organophosphate and carbamate insecticides that produce similar effects in bird embryos do so by lowering levels of NAD, thereby lowering the cells' ATP and energy levels. Rogers et al. (*44*) demonstrated that the effects of organophosphate insecticides in chick embryos could be counteracted by adding NAD. It is possible that the same mechanisms of action are operative in the teleost embryo as well. The looping of the heart has been shown to be caused by shape changes of the heart cells (*45*), and lowered energy levels may not permit this energy-requiring process to take place. Many of the toxicants used in the experiments above are also inhibitors of cell growth (*16*), or mitotic inhibitors (*46*). This growth inhibition can be responsible for the failure of the heart tube to thicken properly, which, in turn, is responsible for the failure of circulation and the subsequent he-

mostasis. It is likely that the lack of blood pigment, which is often observed, has a different etiology, involving inhibition of hemoglobin synthesis.

Skeletal Defects

Perhaps the most commonly observed responses in fish embryos are axial malformations, ranging from slight bending in the skeletal axis to the extreme of no axial development at all. Flexures and stunting have been observed in *Brachydanio rerio* treated with ethyl carbamate (*41*), *Pimephales promelas* treated with toluene (*39*), *Oryzias latipes* treated with aflatoxin (*42*), *Fundulus heteroclitus* treated with mercury compounds (*31,32*), *Oryzias latipes* treated with methylmercury (*47*), *F. heteroclitus* treated with water-soluble fractions of oil (*48*), and *S. gairdneri* treated with cadmium (*35*). Stunting and bent axes have been produced in amphibian embryos by exposure to a variety of herbicides and fungicides (*25,49*), or to mercury (*50*).

A number of investigators have noted a lead-induced production of spinal curvatures in various species of fish including *S. gairdneri* (*51*), *B. rerio* (*52*), and *F. heteroclitus* (*31*) (Fig. 2). The nature of these deformities is comparable to those caused by dietary deficiency in vitamin C or in tryptophan; however, providing excess vitamin C did not counteract the effects of the lead in the rainbow trout (*53*). Muramoto (*54*) attributed the Cd-induced vertebral column damage to a decrease in calcium and phosphorus in the bones which weakened them and made them susceptible to curvature by muscle action.

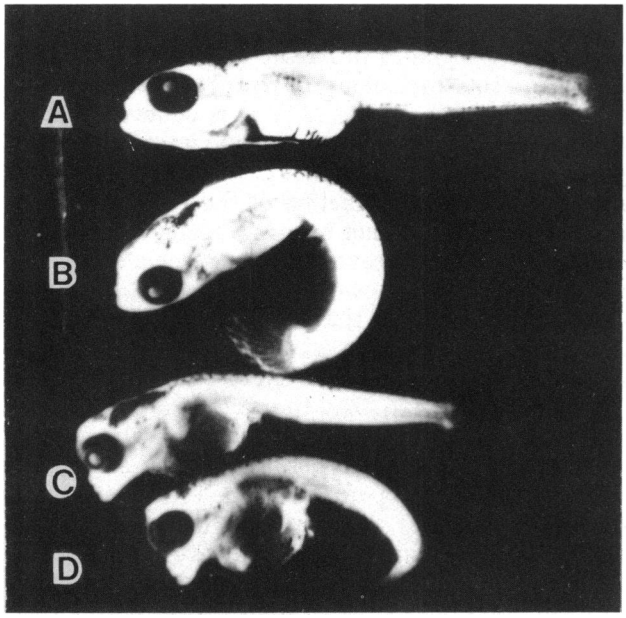

FIGURE 2. One-day post-hatch *Fundulus heteroclitus* larvae (approximately 13×): (*A*) control; (*B–D*) experimental. Such inability to uncurl after hatching has been elicited by lead and by methylmercury. Specimens *C* and *D* also demonstrate synophthalmia. Reproduced from Weis and Weis (*32*).

A failure of axis development was found in Fundulus embryos exposed to actinomycin D, an inhibitor of messenger RNA synthesis (*55*). Despite the failure of axial development, cell differentiation was found to go on, and blood islands, pigment cells, etc., were produced. The fact that cleavage proceeded normally in such embryos indicated that the messenger RNAs for the proteins needed for early development had been made previously by the oocyte and are stable. The new messenger RNAs synthesized after fertilization produce protein products which become important for development after the blastula stage, and their absence in the treated embryos is considered responsible for the developmental defects. Similar studies with a protein synthesis inhibitor, patamycin, produced a specific series of developmental failures, dependent on the time of initiation of the treatment. Defects ranged from a failure of cleavage, through abnormal blastulation, failure of axiation, to anencephaly, depending on the time of initiation of the exposure (*56*).

Very different etiologies may be responsible for skeletal defects produced by some pesticides. Mehrle and Mayer (*57*), studying toxaphene, found that it reduced the amount of collagen in the vertebral column and altered its amino acid composition. The weakened structure was believed to be responsible for the bent condition. Kumar and Ansari (*58*), studying malathion, hypothesized that vitamin C may be responsible for the induction of enzymes for detoxifying pesticides and that the depletion of the vitamin was the immediate cause for the skeletal deformities, since vitamin C is required for polymerization of tropocollagen.

Some pesticides that are neurotoxic may produce skeletal deformities by a physiological rather than developmental mechanism. Kepone (*59*), malathion (*60*), carbaryl (*61*), and other organophosphates (*62*), produce neuromuscular spasms which cause the bent condition. Couch et al. (*59*) observed that the severe spasmodic contortion of the muscle actually broke the centra of the vertebrae in *Cyprinodon variegatus*. Solomon (*61*) demonstrated that the bent condition, which could be produced in *O. latipes* fry by malathion, was reversible, thereby indicating a physiological rather than developmental cause. Observations of neuromuscular disorders are not surprising for insecticides that are acetylcholinesterase inhibitors.

A totally different sort of skeletal anomaly is total or partial duplication of the spinal axis (twinning). This has been observed by Laale (*33*) in ethanol-treated zebrafish, and by Hose et al. (*27*) in benzo[a]pyrene-treated flatfish. Baumann and Sander (*63*), studying the developmental mechanisms responsible for this condition, concluded that cycloheximide and other teratogens delay the proliferation and movement of deep cells more strongly than they delay cell differentiation. The cells, therefore, embark on organogenesis before reaching their final destinations, thereby causing a split in the embryo in the trunk region due to the failure to complete epiboly.

A very subtle skeletal defect is the increase in asym-

metry in the fin ray count in the left versus right pectoral fins. This was observed by Valentine and Soule (*64*) in DDT-exposed grunion, *Leuristhes tenuis*, and the degree of asymmetry was dose-related. These investigators further noted that field collected specimens from a polluted area had higher asymmetry levels than those from a clean area. Asymmetry is similar to meristic variations, which are not considered in this paper, since in both cases it is difficult to distinguish what is normal variation from what is an abnormality.

Pigmentation

A decrease in pigmentation has been noted by some investigators in aquatic species exposed to a variety of toxicants, (*25,47,49,65*). In addition to this general decrease in pigmentation, Ozoh (*65*) noted a disruption in the normal striped pigmentation pattern in the zebrafish, *B. rerio*, exposed to lead.

Interactions

A number of investigators have noted that varying environmental conditions such as salinity can alter the susceptibility of embryos to toxic effects of pollutants. Lowered salinity has been found to increase the toxicity of methylmercury to *F. heteroclitus* (*21*) and to *O. latipes* (*66*). Similarly, decreased salinity increased the toxicity of cadmium to winter flounder (*67*). The enhanced toxicity at lower salinity may be due to greater uptake of water, and therefore, of the toxicants.

Since polluted areas usually involve more than one chemical, it is of interest to analyze the effects of combinations of aquatic toxicants. Thus, there have also been studies of the interactions of two toxicants on developing embryos. Many of such studies with metals have shown antagonisms, in that the toxicity of one metal was reduced by the presence of the other. Zinc and cadmium reduced the effects of methylmercury on *F. heteroclitus* (*21*), silver reduced the toxicity of cadmium to winter flounder (*68*), lead reduced toxicity of copper to zebrafish (*13*), and selenium reduced the toxicity of mercury to medaka embryos (*69*). In the last study, however, the selenium was not effective until the liver had developed in the embryos, indicating an important role of that organ in selenium protection against mercury.

Some studies of combinations of insecticides have shown synergistic interactions. Solomon and Weis (*36*) demonstrated greater than additive effects of low concentrations of malathion and carbaryl in the medaka, *O. latipes*, and Koenig (*70*) showed that small amounts of mirex would enhance the toxicity of DDT to embryos of the cyprinodont, *Adina xenica*. In both these studies, however, when higher concentrations were used, no synergistic effects were observed. Zinc and cygon interacted antagonistically at low levels, while they were additive at other levels (*71*). Teratogens with antagonists or synergists may produce different metabolic effects in the embryo, and thereby alter the the induced abnormalities qualitatively or quantitatively (*72*).

Critical Stages

A number of studies have addressed the issue of critical periods in development for the production of anomalies. Weis and Weis (*32*) have shown that gastrulation in *Fundulus* is the critical period for the genesis of craniofacial defects by methylmercury. This corresponds to the time of induction of the forebrain, defects which are believed to be responsible for the convergence of the optic cups. Akiyama (*73*) identified early cleavage and the time of brain and optic vesicle formation as the times of highest susceptibility of *O. latipes* to mercury. Sharp and Neff (*41*) found that the duration of exposure to mercuric chloride was important in the genesis of spinal curvature in the killifish, *F. heteroclitus*, but identified no critical period. Stoss and Haines (*38*) identified gastrulation as the critical period for the production of malformations in *O. latipes* by toluene. However, in studying carbaryl-induced heart anomalies in *O. latipes*, Solomon and Weis (*36*) found no specific critical period. Even when the complete four-chambered heart had developed, the insecticide could still reduce heart size and bending, produce edema and oscillation of blood flow. Thus, it seemed that the insecticide affected variability of cells rather than developmental processes.

Quantification of Effects

Most reports of teratogenic effects present the data in a qualitative way, describe the defects, and give a percentage of embryos affected. However, in comparing effects of different chemicals or responses of different populations of the same species, it is useful to have a more quantitative approach to the effects. Since many malformations can be more or less severe, it is possible to devise indices to rank embryos in terms of how severely they are affected. Anderson and Battle (*75*), for example, rated chloramphenicol-treated zebrafish embryos on a three-part scale. More refined indices of craniofacial, cardiovascular, and skeletal anomalies produced by methylmercury have been devised (*32,76*) which can give a quantitative estimate of the severity of the defect (Fig. 3). This allows for much more detailed and precise analysis than simply reporting the percentage of embryos affected.

Using indices described above, we have noted striking population differences in susceptibility of *Fundulus* embryos to teratogenic effects of methylmercury (*77*). Embryos from a polluted environment were much less affected than those from a more pristine environment. Possible mechanisms for the increased resistance are a more rapid development time and a less permeable chorion (*22*). Another possible mechanism that has been investigated is the possibility that metal-binding proteins, e.g., metallothionein, which protect against toxic effects of metals by sequestering them, might be found in higher amounts in the embryos from the polluted

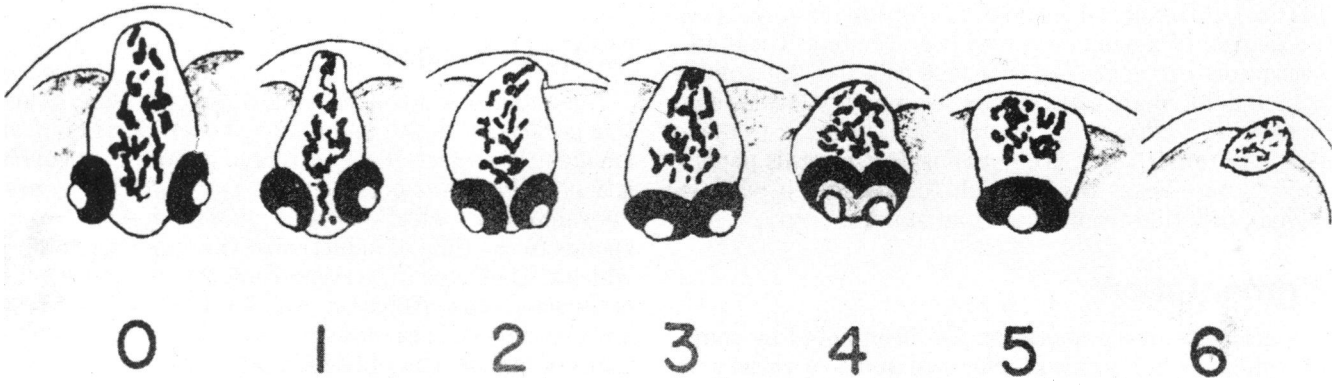

FIGURE 3. Heads of *Fundulus heteroclitus* embryos treated with 50 μg/L methylmercury, demonstrating the 6-part craniofacial index, in which 0 is normal, 1–5 are degrees of convergence of the eyes leading to cyclopia, and 6 has no discernable cranial structure. Reproduced from Weis et al. (*76*).

population. However, significant amounts of this protein were not found until late developmental stages, well past the period of genesis of the malformations (*78*).

Regeneration

Regenerative processes are akin to embryonic development in that morphogenesis and cell differentiation must occur in order to replace the missing structure. These processes can be affected by environmental toxicants. When amputation of a limb or fin occurs, the first process to take place is wound healing. This is followed by the formation of a regeneration bud, or blastema, which is produced by dedifferentiated cells at the site of the amputation (Fig. 4). Wound healing and blastema formation must occur before regeneration proper, and if they are retarded by toxicants, the initiation of regenerative growth will be delayed. By means of analysis of covariance, it is possible to separate out effects of a chemical on blastema formation from those on the growth of the regenerate. Wound healing and blastema formation will affect the intercept, while regeneration itself will affect the slope of the growth curve. Limb regeneration in newts has been observed to be retarded by cadmium (*79*), methylmercury (*80*), and the fungicide Maneb 80 (*81*). In the last study, the growth retardation was ascribed to vascular disturbances that prevented the cell contacts necessary for blastema formation. Fin regeneration in fish has been observed to be inhibited by DDT, malathion, parathion, carbaryl (*82*), PCBs and fuel oil (*83*), cadmium (*84*), and methylmercury (*85*). The Cd-treated fins also exhibited vascular disturbances that may have been responsible for the diminished growth. The reduction of regenerative growth caused by methylmercury may be related to its action as a mitotic inhibitor (*46*).

Fish treated with zinc showed a dose-dependent acceleration of regeneration rate (*86*). Both wound healing and regeneration rate proper were accelerated. Zinc is a trace nutrient requirement which counteracts reduced DNA synthesis (*87*). This effect may be responsible for the observations on regeneration. Acceleration of growth has been observed in other systems at concentrations of toxicant below those which cause retardation. This growth stimulation by low levels of toxicants, termed "hormesis," is believed to result from overcompensation by homeostatic control mechanisms (*88*). Since all zinc concentrations tested caused acceleration of regeneration, it cannot be known whether or not this

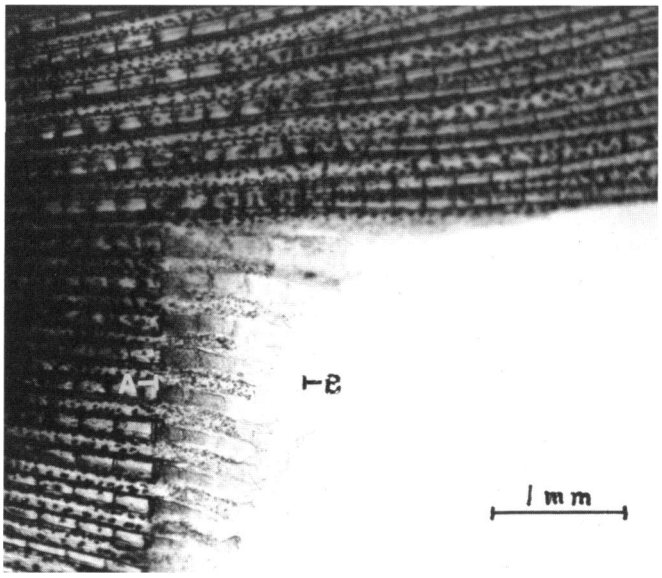

FIGURE 4. Microphotograph of regenerating caudal fin of *Fundulus heteroclitus* one week after amputation. Measurements are made from A to B.

is an example of hormesis. However, cadmium can cause hormesis in regenerating fins of fish that were previously pre-exposed to low concentrations (*89*).

Advantages of Using Aquatic Species

Testing developmental effects of toxicants on aquatic species rather than mammalian embryos has a number of advantages. The organisms are much less costly to obtain and to maintain, and can often be collected from the natural habitat. Their embryonic period is generally shorter, so that data can be collected more rapidly. There is a general, though not absolute, correlation between substances that are teratogenic in aquatic animals and those that are teratogenic in humans. But then, there is also not a 100% correlation between substances teratogenic in mice and rats and those teratogenic in humans. Even among fish, substances that are embryotoxic or teratogenic for one species may be tolerated by another species at much higher concentrations. Predictions of biological effects on a given species cannot necessarily be made on the basis of studies on another species. Nor can predictions of effects on embryos be extrapolated from effects on adults. Large numbers of embryos can be obtained from many fish species. This can enable more in-depth analysis of the data. It is easy to study differences among females in the susceptibility of their embryos to toxicants (*75*) and to investigate population differences in resistance to particular teratogens (*77*). Perhaps the greatest advantage of using aquatic species in teratology studies, however, is the fact that there is no uterus and female animal separating the developing embryos from the eyes of the investigator. One can examine the developing embryos whenever one wishes, make detailed observations on the structure and functioning of the living embryos, and follow the progress of a developing abnormality.

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